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O. Chapleur, Raphaël Civade, Carolina Hoyos-Hernandez, Laurent Mazéas, T. Bouchez. Growing concentrations of phenol increasingly modify microbial communities' dynamics and performances' stability of anaerobic digesters. 13th World Congress on Anaerobic Digestion: Recovering (bio) Ressources for the World, Jun 2013, Santiago de Compostella, Spain. hal-01118701

HAL Id: hal-01118701

<https://hal.science/hal-01118701v1>

Submitted on 19 Feb 2015

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Growing concentrations of phenol increasingly modify microbial communities' dynamics and performances' stability of anaerobic digesters

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Abstract

Anaerobic degradation requires a complex network of interacting and competing microorganisms. Waste anaerobic digesters are based on the intensive use of this flora. Consequently, functioning and stability of digesters are directly related to microbial populations' dynamics. The latter may be subject to external disturbances, such as the arrival of micropollutants with waste, causing malfunction of bioprocesses. In this context, we questioned the influence of phenol addition on microbial communities degrading cellulose under anaerobic conditions. For that purpose, cellulose batch digesters were set-up and inoculated with municipal solid waste digester sludge. Modifications of microbial dynamics and degradation performances after addition of phenol were evaluated in triplicates (nine different concentrations tested, from 0 to 4g.L⁻¹). Gas production, reaction pathways and phenol concentration were followed over time. These results were linked to the monitoring of microorganisms' dynamics (automated-ribosomal-intergenic-spacer-analysis method, ARISA and qPCR on functional gene and on total *Archaea* and *Bacteria*) and of their spatial organisation (fluorescent-in-situ-hybridization, FISH). With increasing phenol concentrations, increasing disruption of digesters' performances (gas production, intermediates accumulation) was observed. EC50 (half-maximal effective-concentration) values for phenol was calculated from the dose/effect curve obtained by plotting degradation parameters as a function of phenol concentration. With increasing concentration, intermediates pathways were modified; *Archaea* activity was totally inhibited from 1.5g.L⁻¹, *Bacteria* activity from 2.0g.L⁻¹. Moreover, phenol degradation was observed in several digesters after several weeks. ARISA monitoring revealed an important influence of phenol concentration on microbial dynamics in general. qPCR gave more specific information. Physico-chemical and microbial results were linked to establish patterns of communities' perturbation by phenol. A phenol inhibition threshold was established for several microorganisms. Subdominant microorganisms that used phenol perturbation to settle and phenol degrading microorganisms were identified. This study gives important insights in the understanding of micropollutants effect on complex microbial communities' dynamics and their influence on digesters' stability.

Keywords

Anaerobic digester, cellulose, fingerprinting, microbial dynamics, micropollutants, phenol, stability

INTRODUCTION

Anaerobic degradation requires a complex network of interacting and competing microorganisms. Waste anaerobic digesters are based on the intensive use of this flora. Consequently, functioning and stability of digesters are directly related to microbial populations' dynamics. The latter may be subject to external disturbances, causing malfunction of bioprocesses. Malfunctions can have significant economic and environmental consequences. For optimal management, stable bioprocesses are thereof highly desirable. Various factors may oppose it. Many chemical compounds, such as heavy metals, sulfides, volatile fatty acids, ammonia, halocarbons and cations can inhibit anaerobic digestion or methanogenesis (Chen *et al.* 2008). Organic pollutants can also be found in a digester and have a negative effect on micro-organisms ensuring anaerobic digestion (Chen *et al.* 2008). In this context, this work was focused on the influence of phenol addition on the microbial dynamics and stability of anaerobic digesters degrading cellulose.

METHODS

Batch system digester and inocula

Anaerobic batch digesters (150 ml) were set up using sludge from a stable laboratory 10 L mesophilic digester degrading cellulose (inoculated from a full scale household waste digester). 100 ml of sludge and 2.7 g.L⁻¹ of cellulose were introduced in each digester. Phenol was added in order to reach 9 different concentrations ranging from 0 to 4 g.L⁻¹ (see table 1). A microcosm without phenol and without cellulose was used as negative control. All the incubations were performed in triplicates. Bottles were hermetically sealed with a septum and headspaces were flushed with nitrogen. The reactors were incubated under anaerobic conditions at 35°C, without shaking. Liquid samples of 4 ml were regularly collected through the septum and centrifuged. Resulting pellets and supernatant were used for biomass and chemical parameters analysis, respectively.

Table 1. Cellulose and phenol concentrations in bioreactors settled for the experiment (triplicates)

Phenol concentration (g.L ⁻¹)	0.00	0.00	0.01	0.05	0.10	0.50	1.00	1.50	2.00	4.00
Cellulose concentration (g.L ⁻¹)	0	2.7	2.7	2.7	2.7	2.7	2.7	2.7	2.7	2.7

Physico-chemical analysis

Biogas accumulation in the headspace was measured and head-space gas analysis was performed every two days as described in (Chapleur 2012). These data were used to calculate gas production and composition. Dissolved organic carbon (DOC), as well as concentrations of acetic, propionic, butyric, isobutyric, lactic and formic acids produced during cellulose degradation, were measured as described in (Chapleur 2012). Phenol analysis was realized with head space - solid phase micro extraction – gas chromatography mass spectrometry technique (HS-SPME-GCMS) technique as described in (Limam *et al.* 2010)

DNA extraction, Polymerase chain reaction (PCR) and Automated Ribosomal Intergenic Spacer Analysis (ARISA)

Total DNA was extracted with the Power Soil DNA Isolation Kit (Mobio Laboratories Inc. Carlsbad). Modifications of archaeal and bacterial dynamics were evaluated with ARISA (Fisher & Triplett 1999) fingerprinting technique as described in (Chapleur 2012). Obtained diversity profiles were statistically compared with the StatFingerprints R package (Michelland *et al.* 2009). Proximity dendrograms were built with Ward algorithm based on Euclidian distance between profiles.

Half maximal effective concentration (EC50)

Amongst others, cellulose degradation inhibition by phenol was evaluated by calculating the EC50 values by plotting initial rate of methane production for the different incubation as a function of initial phenol concentration. The curve was fitted to the Hill model by non-linear regression using the algorithm of Marquard and a non-linear regression program proposed by Duggleby as described in (Fechner *et al.* 2010) to calculate EC50.

RESULTS AND DISCUSSION

Gas production

Total gas production was compared for all incubations as shown on figure 1A. Except for the incubations with 1.50 g.L⁻¹ of phenol, good replicates are observed. A weak biogas production measured for negative controls corresponds to the degradation of organic matter contained in the inoculum. The biogas production of the incubations without phenol (afterwards called positive controls) reaches 70 mg of C after 40 days. The same level of production is observed in the incubations with 0.01, 0.05, 0.10 mg.L⁻¹ of phenol. Phenol has no influence on biogas production at this concentration or was degraded too quickly (figure 1B). For the incubations with 0.50 and 1.00

g.L^{-1} of phenol, gas production is divided in two steps. The first one corresponds to cellulose degradation and is slightly less important than positive controls' production. The second one probably corresponds to the degradation of phenol that is totally metabolized in these incubations after 40 and 54 days (figure 1B). The production of incubations with 1.00 g.L^{-1} of phenol reaches 70% of the production of positive controls after 60 days, and phenol is not degraded yet. Good replicates are observed. A second biogas production is then observed for these digesters, but is not concomitant in all replicates, starting respectively at days 76, 105 and 145. This production is correlated with phenol degradation as shown in figure 1B. The incubations with 2 g.L^{-1} of phenol exhibit a biogas production barely more important than negative control. Finally, nearly no biogas is produced in incubations with the highest phenol concentration. With increasing concentrations of phenol, slower kinetics and lower level of biogas production are observed. An EC_{50} value of 1.40 g.L^{-1} was calculated based on the initial rate of methane production (not shown). Beyond this value, cellulose degradation to biogas is deeply influenced and inhibited, and digesters cannot resist to the perturbation, at least methanogenesis is disturbed.

Intermediates production and accumulation (not shown)

In the incubations with a phenol concentration lower than 1.00 g.L^{-1} , DOC values are stable ($\sim 200 \text{ mg}$ of C per L). Acetic acid is formed but immediately degraded. At 1.00 g.L^{-1} of phenol, a propionic acid accumulation is observed between days 16 and 29 (69 mg of C per L), but propionic acid is finally degraded. An acetic acid accumulation (600 mg of C per L) is then observed at day 61. It probably results from phenol metabolites degradation. At 1.50 g.L^{-1} of phenol, a propionic acid accumulation is also observed between days 9 and 48 (125 mg of C per L). Acetic acid accumulates at 250 mg of C per L between days 0 and 21 for all replicates, (cellulose degradation). A second accumulation is observed, at different dates according to the replicates and accordingly to the observations made on biogas production. In 2.00 g.L^{-1} digesters, both acetic and propionic acids accumulate (respectively from days 0 and 9) but are never degraded. In 4.00 g.L^{-1} digesters no propionic acid is produced and a very low level of acetic acid accumulates (40 mg of C per L). Propionic acid accumulation often indicates a disturbance in anaerobic digestion processes. In our experiment, it occurs from 0.50 g.L^{-1} of phenol, and increases with level of phenol concentration. At the highest concentration, processes are so much disturbed that nearly no VFA production is observed, all the steps of anaerobic digestion are disturbed.

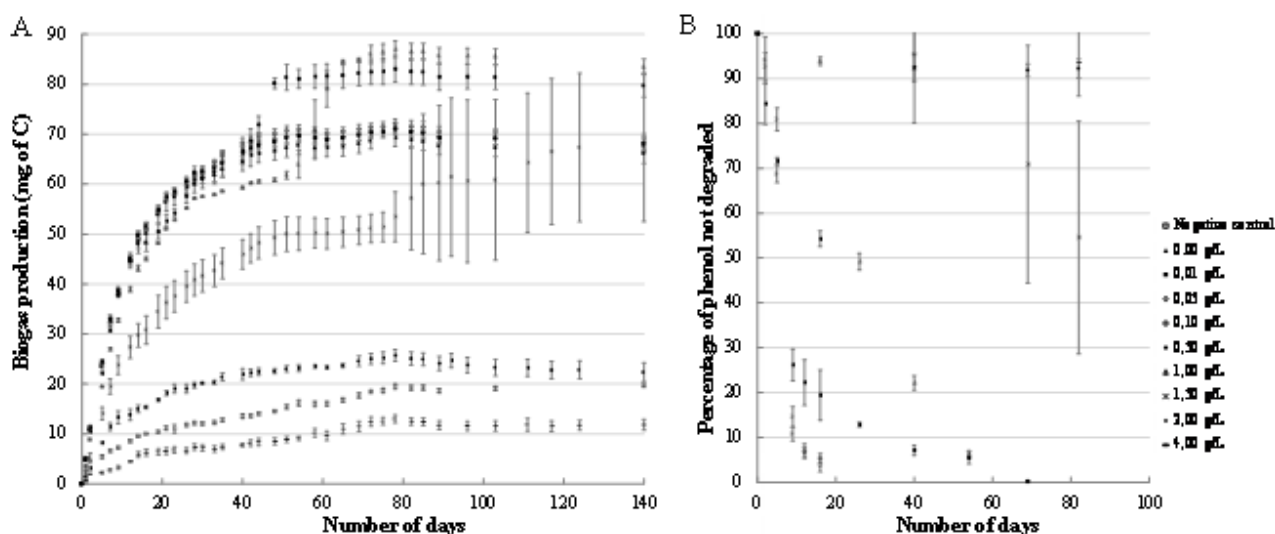


Figure 1. Total Biogas production in mg of C (A) and percentage of phenol not degraded (B) for the different triplicates digesters (mean values, error bars represent standard deviations).

Population dynamics

Statistic comparison of diversity profiles realized over time enabled to build proximity dendrograms for *Archaea* (not shown) and *Bacteria* (figure 2). These results were linked to physic-chemical

parameters. Three main groups of profiles can be distinguished (I, II and III), that correspond to microbial communities moderately perturbed, not perturbed, and strongly perturbed by phenol. These main groups are subdivided in 12 groups (M to X) sharing similar patterns of biogas production, intermediates accumulation and actual phenol concentration. Among these groups, a phenol inhibition threshold was established for several microorganisms identified by sequencing. qPCR on functional genes and total *Archaea* and *Bacteria* supplemented these data (not shown). *Archaea* activity was totally inhibited from 1.5 g.L⁻¹ and *Bacteria* activity from 2.0 g.L⁻¹. Subdominant microorganisms that used phenol perturbation to settle and phenol degrading microorganisms were also identified (not shown).

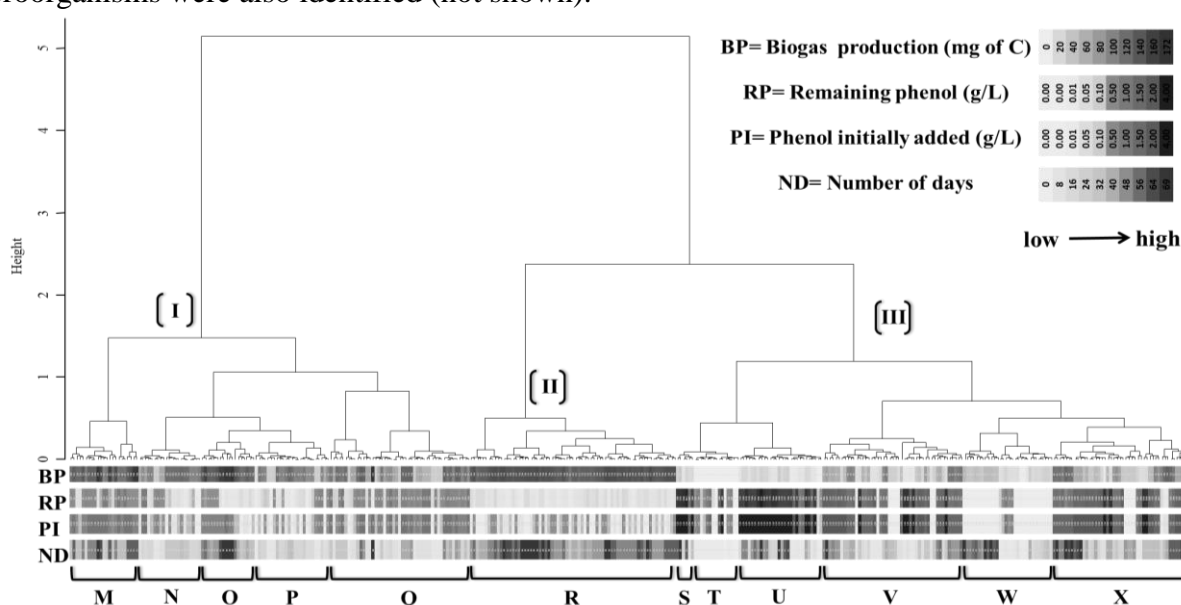


Figure 1. Proximity dendrogram of bacterial ARISA profiles realized during the experiment. For each branch of the dendrogram, number of days of incubation, biogas production, and remaining and initial phenol concentrations are presented.

This study gives important insights in the understanding of micropollutants effect on complex microbial communities' dynamics and their influence on digesters' stability.

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